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Mutation in the gene encoding *1-aminocyclopropane-1-carboxylate synthase 4* (*CitACS4*) led to andromonoecy in watermelon

Summary Although it has been reported previously that ethylene plays a critical role in sex determination in cucurbit species, how the andromonoecy that carries both the male and hermaphroditic flowers is determined in watermelon is still unknown. Here we showed that the watermelon gene *1-aminocyclopropane-1-carboxylate synthase 4* (*CitACS4*), expressed specifically in carpel primordia, determines the andromonoecy in watermelon. Among four single nucleotide polymorphism (SNPs) and one InDel identified in the coding region of *CitACS4*, the C364W mutation located in the conserved box 6 was co-segregated with andromonoecy. Enzymatic analyses showed that the C364W mutation caused a reduced activity in CitACS4. We believe that the reduced CitACS4 activity may hamper the programmed cell death in stamen primordia, leading to the formation of hermaphroditic flowers.

It has been reported that 1-aminocyclopropane-1-carboxylic synthase (ACS), a key enzyme for ethylene biosynthesis, is important for sex determination in several *Cucurbitaceae* species (Boualem et al. 2008, 2009). In cucumber and melon, mutations that cause reduced ACS activities lead to andromonoecy (Boualem et al. 2008, 2009). Modern watermelon (*Citrullus lanatus*) varieties have three common sex forms: monoecious (carrying both male and female flowers), andromonoecious (carrying both male and hermaphroditic flowers) and gynoecious (carrying female flowers only) (Ji et al. 2015), and a recessive locus (*a*) is associated with the andromonoecy (Ji et al. 2015). Eight *ACS* genes, *Cla014652* (*CitACS1*), *Cla014057* (*CitACS2*), *Cla006634* (*CitACS3*), *Cla011230* (*CitACS4*), *Cla000483* (*CitACS9*), *Cla011522* (*CitACS10*), *Cla022653* (*CitACS11*), and *Cla006245* (*CitACS12*) are present in the watermelon genome (Guo et al. 2013, 2015). Four genes in ACS family, *CitACS1*, *2*, *3*, and *4*, were speculated to associate with sex determination (Salman-Minkov et al. 2008; Prothro et al. 2013; Guo et al. 2015). Homology analyses showed that the CitACS4 shared 94% sequence identity with the CmACS-7 in melon at the amino acid level (Figure 1A). The 1,720 bp genomic region of *CitACS4*, containing three exons and two introns (Figure S1), encodes a polypeptide with 444 amino acids (Figure 1A). Therefore, it has been proposed previously that *CitACS4* in watermelon is the ortholog of *CmACS-7*, and might be the candidate gene for the *a* locus in watermelon (Prothro et al. 2013). To address this possibility, we used polymerase chain reaction (PCR) to amplify the genomic sequences of *CitACS4* from 25 watermelon germplasm collections with different sex forms: 19 monoecious, one gynoecious and five andromonoecious (Figure S1). This study enabled us to identify in total five polymorphic sites with either SNPs or insertion-deletion (InDels) in *CitACS4*. Among them, only the SNP of G1477C was co-segregated with the andromonoecy (Figure S1). The mutation caused a cysteine (C) to tryptophan (W) substitution at the residue 364 (named C364W; Figure 1A). We constructed a F_2 population from a cross between the andromonoecious line AKKZW (*aa*) and the monoecious line XHB (*AA*) to test if the C364W substitution was associated with andromonoecy. A dCAPs marker dCAPs_FspBI was designed to detect the polymorphism of G1477C. Genotypic analyses in a population of 440 F_2 progenies showed that all 107 andromonoecious plants obtained were co-segregated with the *aa* genotype of dCAPs_FspBI, while none of the remaining 333 progenies showed andromonoecy, suggesting a tight link of the C364W substitution with the *a* locus (Figure S2). ACS protein has 12 conserved boxes (Rottmann et al. 1991), and the C364W is located in the box 6 (Figure 1B). It has been showed previously that the G33C, P209S and S399L mutations in cucumber ACS and the A57V mutation in melon ACS exhibited reduced ACS enzymatic activities, and plants carrying these mutations showed andromonoecy in flowers (Boualem et al. 2008, 2009).

To determine if the C364W mutation had compromised the enzymatic activity of ACS, three constructs, *His6-CitACS4*, *His6-CitACS4*^{C364W} and *His6-CmACS-7*, were made and transformed into *Escherichia coli*. Fusion proteins were affinity-purified using Ni columns. The ACS enzymatic activity was measured using a buffer containing 10 μM pyridoxal 5′-phosphate (PLP) and 200 μM *S*-adenosyl methionine (SAM) (Boualem et al. 2008, 2009). Results obtained showed that the ACS activity of *His6-CitACS4* was similar to that of *His6-CmACS-7*, while the ACS activity of *His6-CitACS4*^{C364W} was significantly reduced, suggesting that the C364W mutation has compromised the enzymatic activity of CitACS4 (Figure 2A). The 3- dimensional modeling for CitACS4, which referred to the LeACS8 structure in tomato (Huai et al. 2001), showed that the C364 residue is located in the α-carboxylate backbone (Figure S3A, B). In this model, the cysteine residue possesses a mercapto group that can potentially form a disulfide bond to maintain the protein stability (Figure S3B). The C364W substitution may have disrupted the stability of CitACS4, and subsequently damaged the activity of the enzyme.

To examine the expression pattern of *CitACS4*, total RNA was extracted from different parts of watermelon plant. Quantitative real-time PCR assays were performed and results showed that *CitACS4* was expressed specifically in female and Herhap flowers (Figure 2B). According to the stages defined for flower development in cucumber (Bai et al. 2004), *CitACS4*

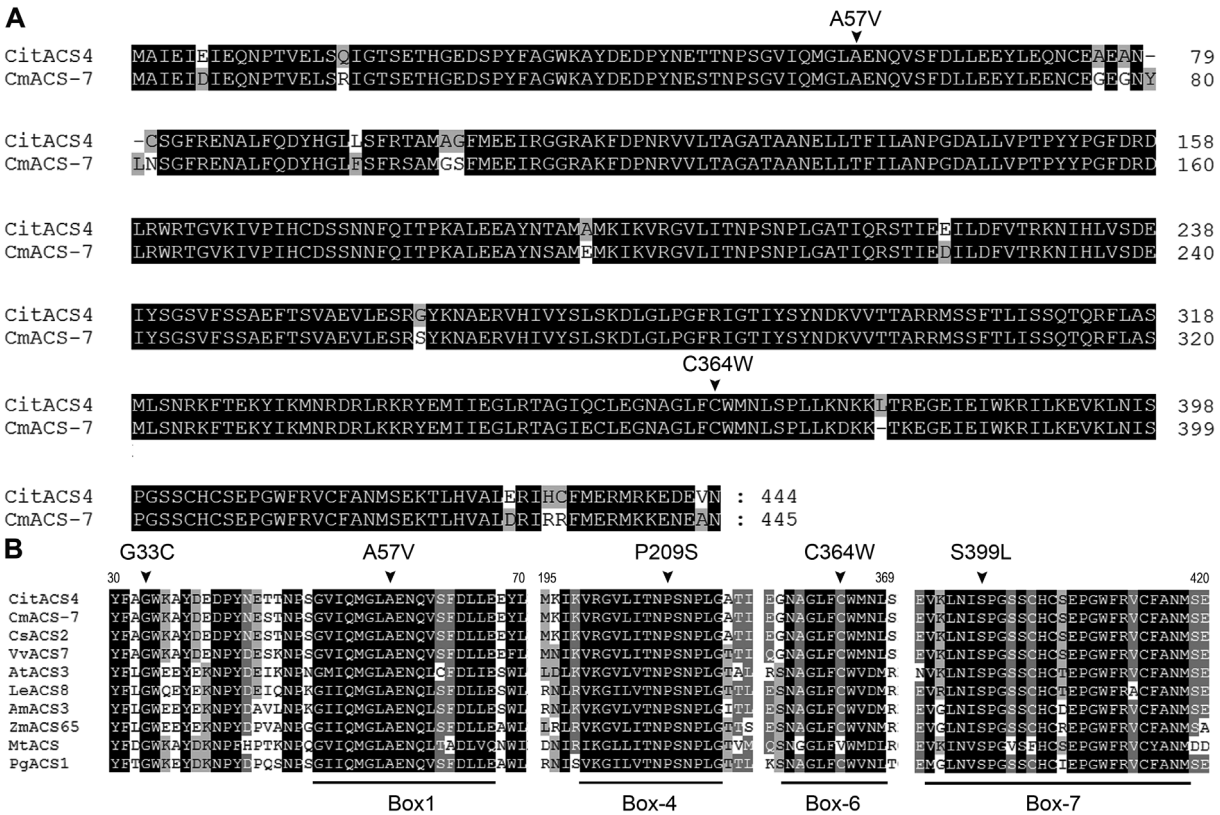

Figure 1. A mutation in a conserved site of CitACS4 is associated with andromonoecy
(**A**) Alignment of CmACS-7 and CitACS4 proteins. The A57V and C364W indicate the amino acid changes associated with the andromonoecious genotype in melon and watermelon, respectively. (**B**) Alignments of CitACS4, CmACS-7, CsACS2 and homologus proteins from AmACS3 (*Antirrhinum majus*, AAC70353), AtACS3 (*Arabidopsis thaliana*, AF322390), LeACS8 (*Lycopersicon esculentum*, AF179247), MtACS (*Medicago truncatula*, AAL35745), PgACS1 (*Picea glauca*, ABM60747), VvACS7 (*Vitis vinifera*, CAN66901) and ZmACS65 (*Zea mays*, AAR25560). G33C, P209S and S399L are mutations in CsACS2, A57V is the mutation in CmACS-7, and C364W is the mutation in CitACS4. All these mutations lead to andromonoecy.

was expressed primarily in stage 6 female and hermaphroditic flower buds, and lower levels of expression were detected in stage 10 and 14 flowers (Figures S4, S5). RNA *in situ* hybridization analysis revealed that *CitACS4* was expressed specifically in the carpel primordia of female and hermaphroditic flower buds at stages 5 and 6 (Figure 2C, D), while no expression was detected in male flower buds (Figure 2E). The *CitACS4* expression pattern is similar to that of the *CsACS2* in cucumber, and the *CmACS-7* in melon (Boualem et al. 2008, 2009). All primers used in this study were listed in Table S1.

The production of ethylene in female floral primordia, mediated by the ACS activity, triggers the programmed cell death (PCD) in male floral organs (Bai et al. 2004). In this study we showed that the andromonoecious sex form in watermelon was caused by a recessive mutation in *CitACS4*. The compromised enzymatic activity of CitACS4 in andromonoecious watermelon varieties may have caused a reduced ethylene production in carpel primordia, leading to the formation of flowers with both male and female organs. This result may potentially be used in breeding and genetic improvement of watermelon in the future.

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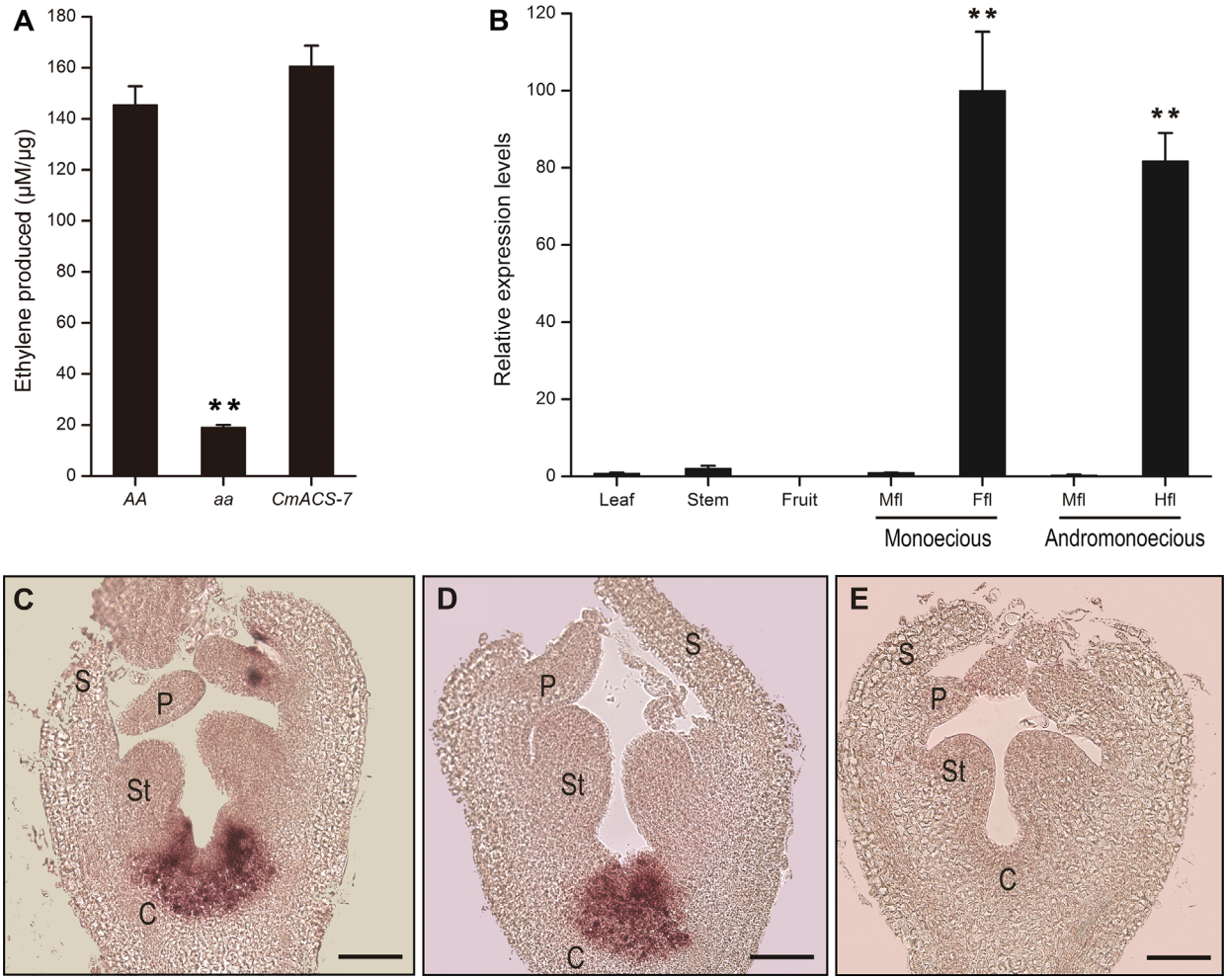

Figure 2. Enzyme activity and expression analyses of *CitACS4*

(**A**) Enzyme activities of *His6-CitACS4*, *His6-CitACS4*^{C364W} and *His6-CmACS- 7* produced in *Escherichia coli*. Note that amounts of ethylene produced by *CitACS4*^{C364W} is significantly lower (indicated with **) than those produced by *His6-CitACS4* and *His6-CmACS-7*. *AA*: *His6-CitACS4*; *aa*: *His6-CitACS4*^{C364W}; *CmACS-7*: *His6-CmACS-7*. (**B**) Expression analyses of *CitACS4* expressions in different organs. Note that, in stage 6 floral buds, *CitACS4* expressions in female (Ffl) and hermaphroditic (Hfl) flower buds are significantly higher (indicated with **) than those in male flower buds (Mfl) and any other organs tested. (**C**) to (**E**) RNA *in situ* hybridization analyses showing female flower in stage 6 (**C**), hermaphroditic flower in stage 5 (**D**) and male flower in stage 4 (**E**). S, sepal; P, petal; St, stamen; C, carpel. Bar = 100 μm.

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AUTHOR CONTRIBUTIONS

G. J. and J. Z. drafted the manuscript. G. J., H. Z., J. Z. and J. S. performed the experiments. G. J., H. S., S. T., S. G. and Y. R. analyzed the data. G. G., H. S. and J. G. contributed materials. Y. X. designed the experiment, supervised the study, and revised the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Sequence analysis of *CitACS4*

Figure S2. Analysis of AKKZW × XHB F_2 population using the dCAPs_FspBI marker

Figure S3. Superposition of the ACS structure

Figure S4. Expression analysis of *CitACS4* in female flowers

Figure S5. Expression analysis of *CitACS4* in hermaphroditic flowers

Table S1. Primers used in this study